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ELECTRICALLY MEDIATED TRAUMA REPAIR

Annual Report

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We are interested in the way applied electrical fields may alter the responses of tissues to traumatic injury - especially nervous tissue. We have determined that an applied field of a particular polarity and magnitude can facilitate the regeneration of the spinal cord nerve fibers around the region of damage. Moreover, we have determined that such an electrical treatment can induce a recovery of function in a reflexive behavior produced by damage to a particular part of the spinal cord white matter. In the absence of such treatment, this behavioral defect is permanent for the life of the animal. We are also investigating the possibility that similar applied fields may enhance the capability of peripheral nerve fibers to regenerate and in so-doing produce an earlier and possibly more complete behavioral recovery.

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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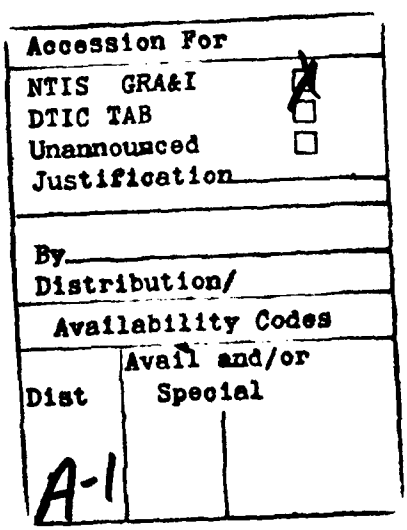

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A. STATEMENT OF PROBLEM

We have recently demonstrated that an applied electrical field can facilitate the regeneration of severed axons within the hemisected spinal cord of adult mammals (Borgens, et al., 1986b). In further studies, in part supported by the DOD, we have demonstrated that such electrical facilitation also is associated with a functional recovery of the CTM reflex (Cutaneous Trunci Muscle), a novel behavioral model system developed in this laboratory for tests of spinal cord functional integrity (Borgens, et al., 1987). Right lateral hemisection of the adult guinea pig spinal cord produces a permanent defect in the functioning of this long tract reflex. There is no recorded incidence of spontaneous recovery of the CTM reflex - the injury and behavioral loss in the skins response to tactile stimulation is indeed permanent for the life of the animal (Borgens, et al., 1987; see also Nixon, et al., 1984). Electrical stimulation produces a recovery of this defect in about 15 - 20 % of the animals tested.

During the last year we have established that:

1. The recovery of the CTM is truly mediated by the effect of the electrical field acting on the central projections of fibers within the spinal cord (and not on the peripheral receptive fields).

2. This recovery of function is dependent on the polarity of the applied field. Functional recovery occurs only in response to rostrally negative fields - not caudally negative fields. The response to a caudally negative field is identical to the sham treated population. This is a satisfying observation since all known responses to imposed fields are mediated by negative electrodes placed in advance of growing or regenerating neurites (reviewed by Borgens, 1989 b and c).

3. Implantation of Matrigel (basal lamina fragments, laminin, and various glycoaminoglycans) into the lesioned spinal cord conforms to the lesion and fills a gap in the spinal cord parenchyma. This material is under testing now to determine if it can provide a substrate allowing axons to regenerate through the region of the astroglial scar - instead their preferred pathway, around it (Borgens, et al., 1986b).

4. Imposed electrical fields do not demonstrate a significant effect on regenerating peripheral nerves (using peroneal nerve preparation ion the guinea pig hind leg) - at least at the field strengths suggested in the literature (reviewed by Borgens, 1989 c).

In the coming year we wish to :

1.) increase our understanding of the nature of the recovery process in the CTM by electrophysiological techniques. This may provide understanding allowing us to enhance this effect.

2.) Determine if an oscillating electrical field will enhance this regeneration and functional recovery as it appears to enhance cultured neurite responses to applied fields (Hinkle, et al., 1981; reviewed by Borgens, 1989 c).

3.) Finish our tests on matrigel and determine if matrigel plus the field will likewise enhance the frequency and character of behavioral recovery in the CTM.

4.) Develop a clinical model for acute spinal cord trauma - the disc herniation induced paraplegia in small dogs. This model will hopefully demonstrate if these laboratory observations of functional recovery in the CTM reflex has clinical relevance to naturally occurring spinal cord injury.

B. BACKGROUND

The basic responses of nervous tissue to applied electrical fields - both in vivo and in vitro - and proposed mechanisms of action of such fields on the cellular level has been reviewed at length in past annual reports. I will not review this historical literature again here, but instead direct the interested reader to recent reviews on these subjects (Borgens, 1988 a and b, 1989 b and c). Here I will review the background information pertinent to our newest studies, that is the CTM as a behavioral index for spinal cord functional integrity; the rationale for the use of various substrates (i.e. matrigel) meant to enhance functional responses to the application of the field; background information on the use of an oscillating electrical field; and finally an introduction to the dog model of spinal cord injury.

1. The CTM as a Behavioral Model for Spinal Cord Injury

The CTM behavior is observed as a phasic rippling of the backskin in response to light tactile stimulation. The reflex is dependent on long tract sensory axons which project rostrally in the ventrolateral white matter of the cord, synapsing on 3 - 4 nuclei of motoneurons within the cervical enlargement. These project motor efferents out of the spinal cord at the level of the brachial plexus, returning to the cutaneous trunchi muscle (panniculus carnosus) of the backskin via the lateral thoracic branch of the brachial plexus. The reflex demonstrates a predominately bilateral organization - that is there is little contralateral responses to stimulation of the skin - there is absolutely no evidence of a contralateral control of ipsilateral motoneuron functioning. Therefore, a right lateral hemisection destroys the reflex on the right side of the cord below the level of the lesion. However, the reflex is intact on the contralateral side and above the level of the lesion, ipsilateral to it. The permanent region of areflexia produced by cord section has never been seen to recover spontaneously (in observations exceeding two years in the guinea pig). In summary, the advantages of the CTM as a behavioral paradigm are:

1. The rippling of the skin is easily quantified using stop-frame video graphic techniques (see Approach and Methodology section).

2. Interruption of the CTM afferent pathway (by spinal cord hemisection) produces a truly permanent defect in behavioral output of the CTM. There is hardly any need for statistical evaluation of the responses

to experimental treatment since all control operations produce permanent defects in the CTM - there is no known propensity of the defect to recover spontaneously.

3. Each animal can serve as its own control, since contralateral CTM responses are unaffected by the ipsilateral hemisection, and CTM responses above the level of the lesion and ipsilateral to it are also unaffected.

4. The anatomy and physiology subserving this reflex pathway is known in much greater detail than more commonly used behavioral models (ambulation or inclined plane tests in rodents and cats) that depend on complex sensorimotor relationships, supraspinal control, and local control of posture and gait.

2. Control of Mammalian Nerve Regeneration by Applied Electric Fields

Work from this laboratory has demonstrated that a distally negative applied field can facilitate regeneration of identified long tract sensory afferents within the spinal cord white matter (rostrally projecting axons of the dorsal columns). These axons circumnavigate the lesion (they do not grow through it) projecting rostrally for several mms (Borgens et al., 1986b). The appearance of the sham treated control spinal cords was unremarkable. The proximal segments of dorsal column axons died back several mm (sometimes 1-1.5 cm) and remained in stasis (Borgens et al., 1986a and b; Gilson and Stensaas 1974). Rarely did these axons even project into the caudal most boundary of the glial scar. Electrically treated spinal cords displayed axons projecting to the plane of transection. Some of these than grew around the lesion bridging to the rostral segment of the spinal cord. We could be certain of these anatomies because we employed a novel marking device (Borgens et al., 1986a) to delimit the boundaries of the dorsal hemisections and since the only axons visualized were those filled several cm caudal to the original plane of section with Horseradish Peroxidase (HRP). (Thus we eliminated the possibility of mistaking regenerated fibers with axons local to the lesion or sympathetic fibers - known to regenerate into cord parenchyma from damaged blood vessels within the spinal cord).

3. Functional Recovery in the CTM

Though we have demonstrated the capacity for an applied electrical field to enhance the regeneration of axons in the dorsal columns - we were unable to test the functional consequences of this regeneration due to the lack of a rigorous behavioral defect associated with dorsal hemisection in the rodent. Therefore, we tested the functional consequences of lateral hemisection and the imposition of an electrical field on the CTM reflex. Twenty-five percent of the experimental population that experienced a rostrally negative electrical recovered CTM behavior below the lesion and ipsilateral to it, while none of the control (sham operated) group demonstrated such a recovery (Borgens et al., 1987). In experiments just completed (and being prepared for submission) we have extended these early findings to include the following;

a.) The opposite polarity of field application does not have any effect on the recovery of the CTM.

b.) The recovery of the CTM is not a transitory recovery - it is persistent for months after it is first observed.

c.) The recovered behavior is a close facsimile to the original CTM phasic rippling of the skin. We have determined this by careful analysis of stop-frame video records of the recovered behavior and its comparison to the phasic rippling recorded prior to spinal cord section.

4. In other studies we have determined that an applied field has no effect on the regeneration of the peroneal nerve (a peripheral nerve of the hindlimb). The response to crush lesions was similar in treated and untreated groups of guinea pigs. This was learned through careful morphometry of nerve cross sections (embedded in plastic and sectioned a 1 um) taken at various positions distal to the original crush at about 2 weeks post lesion. This lack of an enhancement was also mirrored in the behavioral returns in toe spreading behavior, the strength of muscle contraction during twitch tension tests and an analysis of compound action potential propagation through the lesioned nerve at various times post lesion. In electrically treated groups and in the sham treated groups, these responses were all similar. There is some hint in the literature that responses to electrical fields may be more striking when the nerve trunk is severed and anastomosed (Pomeranz, 1986; Roman et al., 1987; Politis et al., 1988). Such lesions are now in testing.

C. APPROACH AND METHODOLOGY

1. Surgical Implantation of the Stimulators

Stimulators are fabricated "in house" and coded, thus the surgeon is blinded to the experimental status of the unit during implantation. Anesthetized animals (35 mg/kg ketamine HCl, 3.5 mg/kg acepromazine maleate, and 5 mg/kg Xylazine) are shaved and surgically draped. The peritoneal cavity is opened and the stimulator is inserted, electrodes left protruding from the cavity. This opening is closed, and the electrodes are routed underneath the skin to the dorsal midline where they exit a midline incision to the backskin. The animal is placed ventral surface down and three laminectomies are performed; one central one, midthoracic level, for the lesioning of the spinal cord. The other two surgical approaches to the cord, one rostral and one caudal by 2 cm, are for the implantation of the electrodes. Electrodes are secured within these laminectomies, with the exposed portion of the electrodes near, but not touching the spinal cord.

2. Stimulator Design

The standard unipolar stimulator has undergone no design changes in the last year, and we refer the reader to a complete description of this unit in the last annual report. We have solidified a design for the oscillating field stimulator in the last year. These began testing in

cases of naturally caused cases of paraplegia in dogs (a new clinical study), and we are miniaturizing these further for implantation into guinea pigs.

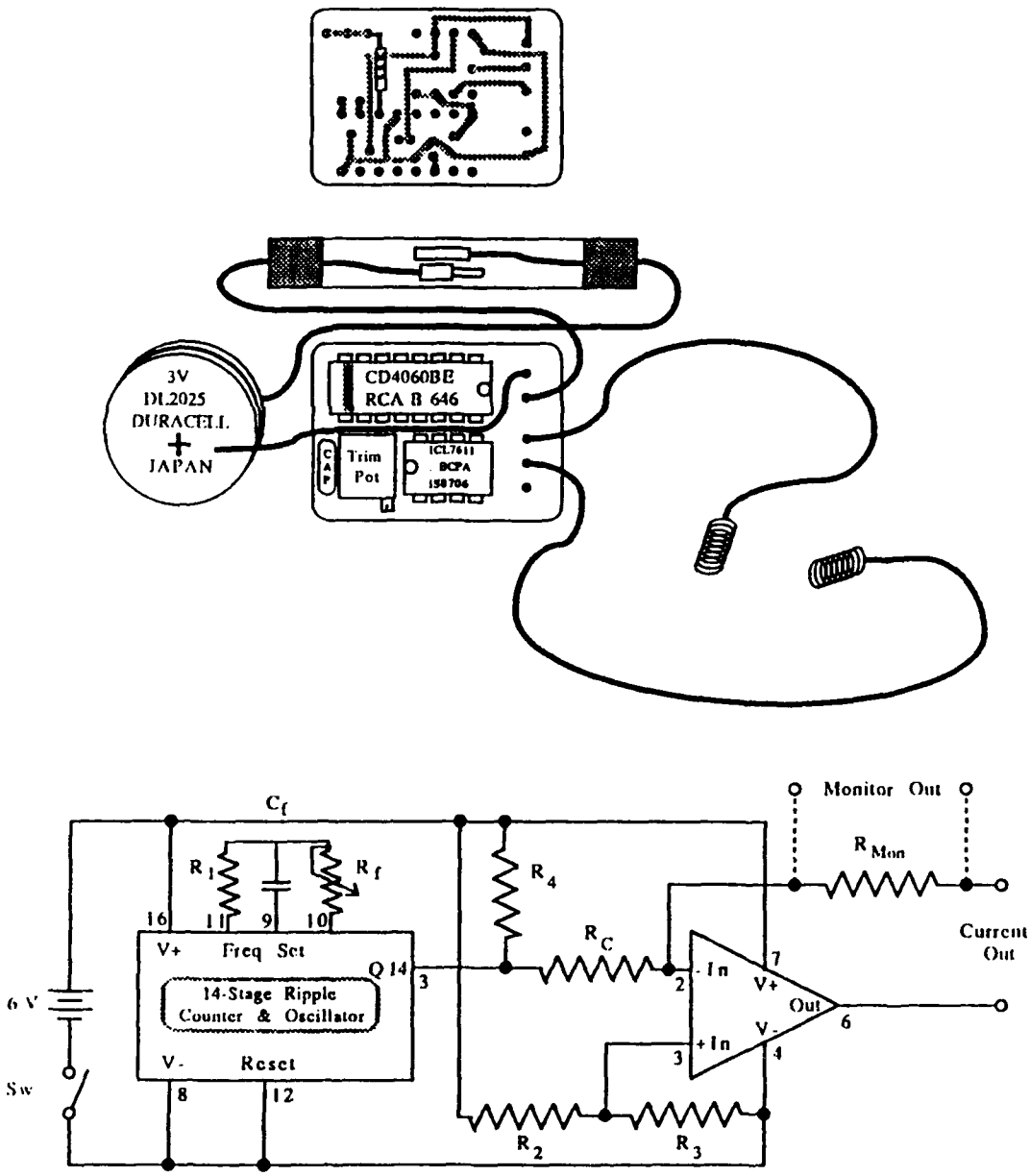

Figure 1. Oscillating Field Stimulator. The circuit has been built on a circuit board, both the top and bottom of which are shown schematically. Most of the resistors are placed underneath the semiconductors. Current output is set by the value of R_c and the frequency of polarity reversal by R_f. The switch consists of a male and female connector encapsulated in a silicone tube. The tube can be stretched to insert the male into the female. The outside of the female connector is covered with heat shrinkable Teflon to prevent contact before it is desired.

The oscillating field stimulator we have designed is capable of delivering a constant current that reverses polarity after virtually any time period desired. The circuit is shown in Figure 1. The timing circuit is based on a CMOS (complementary metal oxide semiconductor) 14-stage ripple-carry binary counter (CD 4060) that contains an oscillator. The frequency of the oscillator is set by a resistor (R_f) and capacitor (C_f) and can be varied from 500 kHz to less than 1 Hz. The output of the oscillator can then be divided by up to 14 binary stages to achieve very low-frequency oscillations (as low as one cycle every 5 hours). For example, we set the oscillator frequency at 4.5 Hz and divide by 14 stage to produce a frequency of 1 cycle/hour (equivalent to reversing the polarity every 30 minutes). To achieve very precise timing, a miniature potentiometer (trimpot, Spectrol model 64X) can be substituted for R_f and adjusted until the desired frequency is obtained. The output is taken from the binary stage desired (stage 14 in our case) as a 0-6-V square pulse (railed at the supply voltage). This voltage is applied to the inverting input of a low-power CMOS operational amplifier (ICL 7611), and a 3-V signal is applied to the non-inverting input (obtained by dividing the supply voltage between R_2 and R_3). This then gives a net + or - 3V at the op-amp input that drives current through the electrodes. The magnitude of the current is determined by the value of R_c by the equation :

$$I = \frac{3V}{Rc}$$

For currents less than 100 uA, the quiescent current of the op-amp is set at 1 uA by strapping pin 8 to the positive supply voltage. To deliver more than 100 uA, this pin should be left floating, which programs the quiescent current to 10 uA. The device draws approximately 65 uA from the battery to supply 20 uA to the tissues. Using the small (12 mm-diameter) BR 1225 lithium dioxide cells (35 mA hours) allows a minimum of 22 days of continuous operation. For longer times or higher currents, we use the larger BR 2025 cells, which have a 120-MA hour capacity.

3. Histological Procedures

Our use of Horseradish Peroxidase (HRP) for the anterograde intracellular filling of axons, the processing of this enzyme to allow visualizing of the insoluble colored by-product, the final clearing of the spinal cord, and the use of the marker device have not changed in the last year. We refer the reader to complete details of these procedures in the last annual report. We have re-investigated the use of the vibratome for the sectioning of spinal cord segments. We began our studies using this device (Borgens et al 1986 a and b), but have since sectioned the cords at about 30um with the cryostat. We believe the older technique to be superior - 50 - 60 um thick Vibratome sections contained more information and made reconstruction of axonal anatomies less difficult. Thus, we are returning to this technique.

Morphometric analysis, procedures for randomizing and blinding, and statistical analysis have not changed since the last annual report. We have however refined our behavioral techniques using the CTM reflex and our use of electromyography to physiologically characterize the reflex.

4. Stop-Frame Video analysis of the CTM

We have refined the computer graphic reconstructions of the CTM by using a new and updated program. This allows complete graphic reconstruction of the video image of the guinea pig,the vectoral movement of dots (tattooed on the animals skin) during skin contraction, and video enhancement of subcompartments of the receptive field. The following figure demonstrates this increased capability.

An analysis of the cutaneous trunci muscle (CTM) reflex was accomplished by first shaving the back of the animal (under light sodium pentobarbital anesthesia - ca. 30 mg/kg, i.p.) and marking the skin with a matrix of india ink dots. Movement of the skin can then be recorded as movements of the dots when filmed with a Panasonic videocamera and NEC D x 1000 u VHS videorecorder positioned above the animals. Tactile stimulation of the skin during these measurements was performed with a 26 gauge hypodermic needle. Computer-graphic reconstructions of these skin movement as well as reconstructions of the receptive fields is performed by using a videographics software package (Magic Paint), a MacIntosh SE computer, and hardcopy rendering via a Laser Writer Plus laser printer (Apple) (Fig. 2 next page).

5. Electromyography

Electromyographic recordings of skin contractions were performed on lightly anesthetized animals. EMGs were recorded from subdermal wire electrodes located at the brachial region near the midline of the back in a region of most visible skin contraction. EMGs were amplified with a Grass P 15 D preamplifier and displayed on either a Tektronix 5113 Oscilloscope or a digital oscilloscope (Nicolet). Permanent records were polaroid photographs of the sweep (made with a Tektronix oscilloscope camera) in the former. In the latter, records were transferred to floppy disk using a Zenith 150 minicomputer in continuity with the oscilloscope. Permanent records could be recalled from the disk by printout on an omniscribe chartrecorder, or plotted on a dot matrix printer. Stimulation of the skin using watchmakers forceps in electrical continuity with the animals and oscilloscope (to produce the stimulus artifact in each record) as in Borgens et al., 1987. In some cases, stimulation of the skin was electrical, using a bipolar stimulation electrode and a Grass S 44 stimulator. All electromyographic recordings were accompanied by a visual scoring of the presence or absence of the CTM reflex. Such observations were made by stimulating the four quadrants of backskin tactually with watchmakers forceps. These quadrants were defined as right flank (below the level of the lesion), right flank (above the level of the lesion), left flank (below the level of the lesion), and left flank (above the level of the lesion). All visual observations and electromyography was performed by a technicians blinded to the experimental status of the animals.

Figure 2. This drawing was made by a computer-graphics integrated system. First the outline of the guinea pig was rendered by a laser printer from a stop-frame video tape image-phased to the graphics analyzer. A dot-matrix pattern was rendered from a videoframe chosen immediately prior to stimulation (note probe) and after stimulation. The closed circle is the position of the dots on the backskin, and the open circle is the position they occupy after CTM skin movement (superimposed by the computer). In this fully recovered animal note that stimulation ipsilateral and below the level of the hemisection once again produces a typical phasic rippling of the skin in response to tactile stimulation. This reflex recovered 121 days after right lateral hemisection in response to a rostrally-negative applied field of about 100 uV/mm.

D. CONCLUSIONS

1. An applied electrical field can facilitate the regrowth of severed spinal cord axons in the adult mammal. There is little evidence that the robust regeneration of peripheral nerves in the legs of rodents following "crush" lesions can be facilitated.

2. Peripheral nerve responses to applied electrical fields may only be observed in response to more severe "cut and anastomose" type lesions.

3. Recovery of function in the CTM long track spinal reflex is modest in frequency (15 - 25%), yet by all analytical methods, quite appropriate in functional character. Quantitative electromyographic evaluation of only two recoveries (and comparison to a larger number of normal animals) suggests that there are indeed physiological distinctions between the transmission and expression of neuromuscular conduction through the reflex.

E. RECOMMENDATIONS

The apparent discrepancy between the ability of an applied field to facilitate the regeneration of CNS but not PNS nerve "crush" lesions needs amplification. It may well be that the robust regeneration, and the early return of function, in crushed peripheral nerve (with most of the facilitation pathways intact) may be near a biological maximum. More severe injuries (such as when the entire nerve trunk is severed and reconnected surgically) may produce conditions where facilitation by an electrical field can be observed and may enhance the rate of the recovery process. We recommend a thorough testing of the effect of distally negative fields upon such severe peripheral nerve lesions. It is such lesions where clinical prognosis in human injuries is less optimistic - additionally, such lesions (severance) may be more commonplace under military conditions (active engagement).

It is now clear that the functional restoration of an otherwise permanent spinal cord defect can be achieved by the application of an applied electrical field. We recommend further investigation at two levels to realize the possible clinical benefits from these laboratory investigations:

1.) at the basic research level: here we need to more completely characterize the anatomical, behavioral, and physiological recovery process; to be able to produce a more optimum response.

2) at the clinical level: to be able to determine if the functional recoveries observed in a focused laboratory behavioral model (the CTM reflex) are really relevant to the many differing defects caused by spinal injury in man. The clinical model we have access to (within the School of Veterinary Medicine) is paraplegia in the dog. Canine paraplegia can result from traumatic injury (fractures associated with car accidents, gunshot wounds) and quite commonly, disc herniation. (Disc herniation in man is a completely separate phenomenon than that in the dog; rarely do human injuries result in spinal cord damage - in the dog, the injury is

exclusively spinal cord mediated. Moreover, the clinical presentation is a perfect model for human paraplegia: permanent loss of continence, loss of volitional movement below the level of the lesion, and loss of superficial and deep pain proprioception. These differing phenomenologies result from different developmental anatomies in dog and man - the cord ends at L 1 in man, L5 in the dog, and dramatic differences in the ligamentous support within the vertebral column predispose human discs to rupture projecting laterally towards the nerve roots, but instead centrally in the dog towards the spinal cord. We have already begun pilot studies in dog paraplegia and requested continuing support from the DOD to more fully explore acute traumatic injuries (especially fracture injuries and gunshot wounds to the spine). Only in this way can we determine the relevance of the laboratory studies (CTM) to clinical medicine and to work out a modality to affect the regrowth of central fibers that project towards the brain as well as descending tracts at the same time and in the same animal.

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GLOSSARY

Dorsal Columns These large spinal cord tracts are bundles of neurons that project into the spinal cord from segmental ganglia lying just outside the cord itself. Sensory information (largely) is carried to the brain by these tracts that ascend the cord.

Laminectomy Surgical exposure of the spinal cord within the vertebral column.

Neurite A general and non-specific term for a neuronal process.

Wick electrode An aqueous "wire". Stimulating electrodes fashioned from a silastic tube, filled with mammalian ringers and a cotton string (the "wick"). Thus, current is carried to the tissues by a conductive solution similar to body fluids and not by metallic wires (which contaminate the tissues with electrolysis products).

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